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Cytotoxic effect of methanol extract of *Conyza bonariensis* on DMBA-induced skin carcinogenesis: An *in vivo* study

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Abstract

In the present study, we examined the cytotoxic effect of *Conyza bonariensis* (methanolic extract). The skin carcinogenesis was induced in two stages, first, applying tumor initiator, 7-12-dimethyl benz(α)antheracene and thereafter applying croton oil, a tumor promotor in Swiss albino mice. The morphological alterations observed and measured during the induction of skin ulceration, included; cumulative number of papilloma, tumor yield and tumor burden. *C. bonariensis* extract (300 and 600 mg/kg/day) was applied locally on mice skin for 16 weeks. The higher dose (600 mg/kg/day) inhibited the tumor formation up to 40% and showed a significant decline in cumulative number of papilloma of continuous group. The results indicated that extract increased the reduced glutathione, superoxide dismutase and catalase, and decreased lipid peroxidation compared to carcinogen group. Histopathological changes showed papilomatosis and ulceration in carcinogen control group. HPLC analysis indicated the presence of flavonoid i.e. quercetin which may be responsible for the cytotoxic action of *C. bonariensis* methanol extract.

Introduction

Cancer is emerging as one of the most horrific disease and almost all of the anticancer drugs available in the market have serious side effects. So, it is direly needed to explore new anticancer agents from plants which can effectively kill cancer cells without damaging normal body cells.

Many plants like *Convolvulous arvensis* (Saleem at al, 2014), *Catharanthus roseus* (Cragg and Newman, 2005; Okouneva et al., 2003; Simeons et al., 2008), *Podophyllum peltatum* and *Podophyllum emodi* (Shoeb et al., 2006), *Taxus brevifolia Nutt, Taxus baccata* (Kingston, 2007; Hait et al., 2007), *Camptotheca acuminate* (Zhang et al., 2004, Fuchs et al., 2006), *Berberis amarensis* (Xie et al., 2009; Xu et al., 2006), *Hydrastis canadensis L* (Wang et al., 2011; Patil et al., 2010), *Tabebuia avellanedae* (Li et al., 2000; De Almeida, 2009), *Betula alba* (Fulda, 2008), *Colchicum autumnale* (Dubey et al., 2008), *Curcuma longa* (Sa et al., 2010; Goel et al., 2008), *Wikstroemia indica* (Lu et al. 2011; Diogo et al., 2009), *Psoralea corylifolia* (Moon et al., 2006; Dixon and Ferreira, 2002), *Ochrosia elliptica* (Kuo et al., 2006), *Amoora rohituka* and *Dysoxylum binectariferum* (Mans et al., 2000), *Euphorbia peplus L.* (Hampson et al., 2005), *Ipomoeca batatas L* (Ancuceanu and Istudor, 2004), *Salvia prionitis* (Deng et al., 2011), *Centaurea schischkinii* (Shoeb et al., 2005) have been known to possess anticancer activity.

In the present study *Conyza bonariensis* was selected to evaluate its cytotoxic efects on DMBA induced skin carcinogenesis. *C. bonariensis* is a cosmopolitan plant and belongs to family Asteraceae (Compositeae). It is used in fungal and bacterial infections (Chaudhry et al., 2001), hepatic toxicity and gastro enteritis, diarrhea, leucorrhoea, menorrhagia. It possesses anticoagulant (Favila and Antonio, 2006), antioxidant (Shahwar et al., 2012), homeostatic, tonic, astringent (Ahmad, 2007),

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cholinergic (Khan et al., 2006), anti-inflammatory and antimitotic activities (Santana et al., 2011). The following study indicated that methanol extract of *C. Bonariensisis* possesses protective effects against skin carcinogenesis in Swiss albino mice.

Materials and Methods

Collection of plant

Whole plants of *C. bonariensis* were collected during April and May from both sides of motorway M2 from Lahore to Islamabad and were identified by a plant taxonomist Dr. Mansoor Hameed, head of Botany Department, Agriculture University, Faisalabad.

Preparation of plant extract

The aerial parts were washed, chopped and dried under shade at room temperature for several days until fully dried, ground by electric grinder, powdered and sieved. The powdered material was macerated in methanol for 7 days with frequent shaking every day, filtered out by using Whatman filter paper. Finally, the solvent from solid material was removed by using a rotary evaporator at 45-55°C and residues obtained were stored in small amber jars at 4°C.

Drug/chemical

Carcinogen, 7-12-dimethyl benz(a)antheracene (DMBA) and croton oil obtained from Sigma-Aldrich Chemical Company USA. Acetone was used as a vehicle for all topically applied carcinogens and dilution of plant extract and methanol (analytical grade) were purchased from Asian scientific store, Jinnah colony, Faisalabad.

Experimental animals

The male mice, 6-8 weeks old, weighing 20-30 g were obtained from National Institute of Health Islamabad and kept in animal house of department of Pharmacology, Government College, University Faisalabad under controlled conditions of temperature (25 ± 1°C) and humidity (50 ± 5°C). They were given standard diet and water *ad libitum*. Mice were acclimatized to environment for one week prior to commencement of experiment (Roslida et al., 2011).

Experimental plan

Dorsal skin of albino mice was shaved with an electric clipper for approximately 2 x 2 cm area and marked with permanent marker (Arya and Kumar, 2011). A total number of 50 animals were selected and divided into 5 subgroups (Figure 1): (Carcinogen control group): 10 mice were applied topically with a single dose of DMBA as a tumor initiator on the shaved area of skin of mice and two weeks later croton oil was applied as tumor promoter thrice a week till the end of 16th week. (Pre group): This group was subdivided into two sub groups, each group consisting of 5 mice and 300 mg/kg and 600 mg/kg *C. bonariensis* (methanol) extract was given topically to these two subgroups for consecutive 7 days. DMBA single topical dose was applied at 8th day and two weeks later croton oil was applied thrice a week till the end of 16th week. (Peri group): Ten mice were divided into two sub groups each having 5 mice and were given DMBA single dose topically, then 300

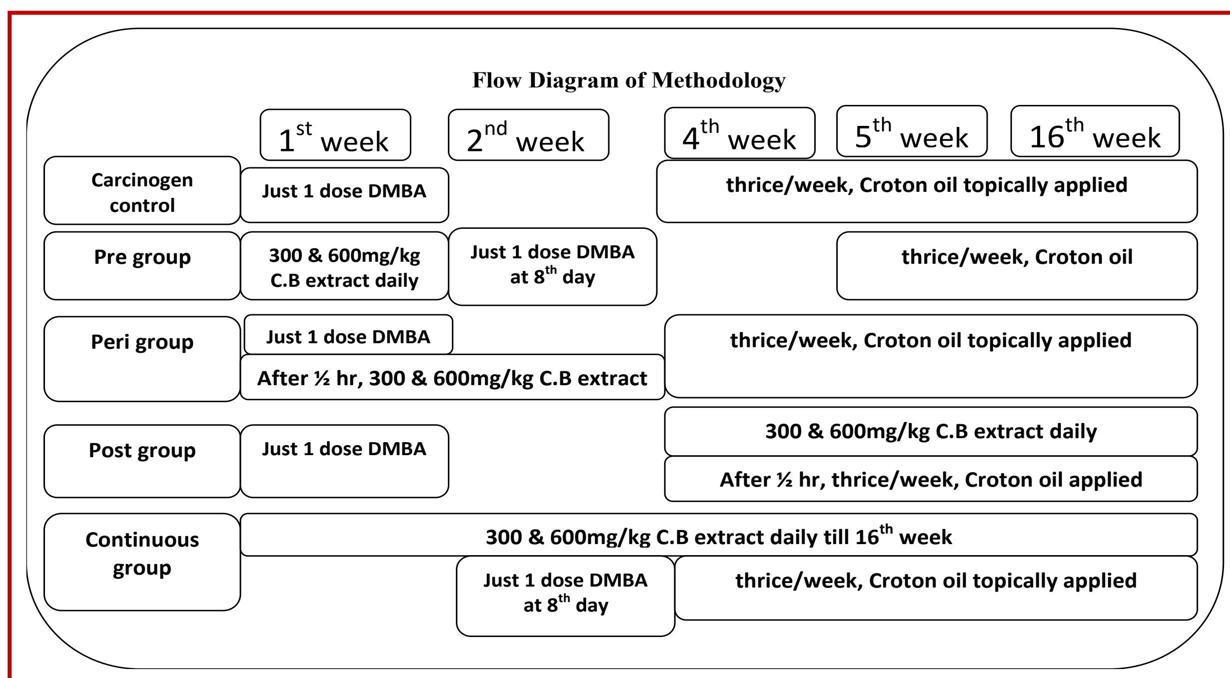

Figure 1: Flow diagram of methodology

mg/kg and 600 mg/kg *C. bonariensis* (methanol) extract topically for consecutive 15 days. After that, croton oil was applied thrice a week till the end of 16th week. (Post group): Initially single dose of DMBA was applied to this group. Then the group was subdivided into two subgroups of 5 mice in each group and after 2 week, first subgroup received 300 mg/kg and second subgroup received 600 mg/kg *C. bonariensis* (methanol) extract topically and half an hour, croton oil was applied thrice a week till the end of 16th week. (Continuous group): Two subgroups, each having 5 mice received 300 mg/kg and 600 mg/kg *C. bonariensis* (methanol) extract topically throughout the experimental period daily and at 8th day, DMBA single topical dose was applied and two weeks later croton oil was applied thrice a week till the end of 16th week.

Preparation of stock solutions

1M DMBA was dissolved in acetone at 100 µg/100 µL (w/v) and croton oil at 1 µg/100 µL to make 1% (v/v) dilution, prepared just before its use and kept in amber glass bottle at about 20°C. A stock solution of extract was prepared by dissolving 10 mg extract in 1.0 mL acetone. Serial dilution of 300 mg/kg and 600 mg/kg was made (Roslida et al., 2011).

Preliminary phytochemical screening

Preliminary phyto-chemical analysis of *C. bonariensis* was performed according to protocol previously described (Mojab et al., 2003; Khan et al., 2011).

Determination of cytotoxic activity

Cytotoxic effect of the methanol extract of *C. bonariensis* was evaluated by considering various morphological parameters like 1- Tumor incidence: number of tumor bearing mice, 2- Cumulative number of papilloma: Total number of papillomas, 3- Tumor yield: average number of tumor per mouse and 4- Tumor burden: number of tumor per tumor bearing mice (Roslida et al., 2011).

Morphological studies

Skin of each mice was weekly observed for loss of hair, redness, ulceration and outgrowths. These were counted and measured by digital vernire caliper till the end of 16th weeks.

Biochemical studies

Mice were sacrificed and shaved ulcerated skin were removed, washed with cold normal saline and kept in formalin bottles. Excised skin was used to prepare 10% tissue homogenate in 0.15 molar tris potassium chloride having a pH of 7.4. Then centrifuge for ten mints at 2000 rpm. reduced glutathione, superoxide dismutase, catalase and lipid peroxidation level were determined by the methods previously described (Marklund and Marklund, 1974; Moron et al., 1979; Ohkhawa et al., 1979; Aebi, 1984).

Histopathological study

Specimens of mice ulcerated skin were excised, washed with normal saline and fixed in 10% formalin for a day. Again fixed with paraffin wax, cut 5 µm portion of each specimen and observed the histopathology (Parmar et al., 2011).

Preliminary phytochemical analysis

Preliminary phyto-chemical screening methanol extract of *C. bonariensis* was performed according to the procedure described elsewhere (Saleem et al, 2014).

Chromatogram by HPLC for identification of active constituent

High performance liquid chromatography (HPLC) was performed to identify various compounds present in *C. bonariensis* methanol extract (Ali et al., 2013). The sample was dissolved in 5 mL distilled water and 12 mL methanol, kept for 5 min, again added 6 mL distilled water, waited for 5 min and added 10 mL 5M HCl in this solution. Placed in oven for 2 hours and filtered the solution by syringe filter. Isocratic: dichloromethane: methanol (60:20:20) was used as the mobile phase with the flow rate of 1 mL/min. The column was ODS 250 mm x 4.6 mm and UV detector was used to obtain chromatogram at 280 nm at room temperature (Saleem et al., 2014).

Statistical analysis

All the obtained results were statistically analyzed by one way analysis of variance (ANOVA). Minitab 16.0 software was used for calculation and all values were represented as mean ± standard deviation (STD). Values were taken as $p<0.001$ (significant).

Results

Results obtained from present study had shown that single topical application of carcinogen DMBA followed by a thrice/week repeated application of 1% croton oil till 16th week produced 100% skin ulceration in the carcinogen control group. 18.2 ± 1.6, 3.6 ± 0.3 and 3.6 ± 0.3 were calculated as the cumulative number of papilloma, tumor yield and tumor burden respectively (Table I). Ulcerated skin specimens were observed under microscope after fixation with 10% formalin, shown in Figure 2.

C. bonariensis (methanol) extract was applied locally to pre, peri, post and continuous groups. Significant decline in cumulative number of papillomas in 300 mg/kg and 600 mg/kg from pre to continuous group 16.8 ± 3.6 to 7.4 ± 4.9 and 15.8 ± 2.4 to 4.8 ± 6.6 respectively. 300 mg/kg extract showed tumor yield from pre to continuous groups 3.4 ± 0.7 to 1.5 ± 1.0 but 600 mg/kg

Table I

Inhibition of morphological parameters of DMBA/croton oil-induced skin tumors by *C. bonariensis* methanol extract

	Control	Pre group		Peri group		Post group		Continuous group	
		300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg
Cumulative number of papilloma	18.2 (1.6)	16.8 (3.6)	15.8 (2.4)	12.0 (2.6)	10.6 (6.2)	10.0 (6.0)	8.6 (5.0)	7.4 ± 4.9^{c}	4.8 ± 6.6^{c}
Tumor yield	3.6 (0.3)	3.4 (0.7)	3.2 (0.5)	2.4 (0.5)	2.1 (1.2)	2.0 (1.2)	1.7 (1.0)	1.5 ± 1.0^{c}	1.0 ± 1.3^{c}
Tumor burden	3.6 (0.3)	3.4 (0.7)	3.6 (0.3)	2.4 (0.5)	2.7 (1.6)	2.5 (1.5)	1.2 (1.7)	1.9 ± 1.2^{c}	1.2 ± 1.7^{c}
Tumor incidence	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	4.0 (2.2)	4.0 (2.2)	4.0 (2.2)	3.0 (2.2)	4.0 ± 2.2^{c}	3.0 ± 2.2^{c}

Data are mean (SD); Significance, $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$

Table II

Induction of reduced glutathione, superoxide dismutase, catalase and inhibition of lipid peroxidase level by *C. bonariensis* methanol extract

	Control	Pre group		Peri group		Post group		Continuous group	
		300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg	300 mg/kg	600 mg/kg
Reduced glutathione	3.3 (0.2)	3.7 (0.4)	7.6 (0.6)	4.1 (0.6)	8.7 (0.9)	7.9 (0.7)	10.3 (1.0)	9.7 (0.7)^{c}	12.1 (1.1)^{c}
Superoxide dismutase	1.7 (0.1)	2.0 (0.6)	4.2 (0.4)	5.5 (0.5)	6.7 (0.7)	7.0 (0.5)	8.8 (1.0)	7.9 (0.6)^{c}	9.1 (0.8)^{c}
Catalase	13.6 (0.8)	13.8 (0.6)	16.0 (0.3)	16.5 (0.7)	18.3 (1.1)	19.6 (1.0)	21.2 (1.3)	18.8 (0.7)^{c}	25.5 (1.0)^{c}
Lipid peroxidation	7.7 (0.2)	6.2 (0.4)	5.8 (0.2)	4.9 (0.5)	3.5 (0.8)	4.1 (0.5)	2.9 (0.6)	4.2 (0.6)^{c}	1.4 (0.4)^{c}

Data are mean (SD); The results are compared by one-way ANOVA (analysis of Variance); Significance, $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$

showed 3.2 ± 0.5 to 1.0 ± 1.3. Tumor burden in 300 mg/kg was 3.4 ± 0.7 to 1.9 ± 1.2 and in 600 mg/kg was 3.6 ± 0.3 to 1.2 ± 1.7 shown in Table I. The tumor incidence in continuous group was compared ($p<0.001$) with carcinogen group decreased up to 20% with 300 mg/kg but 40% with 600 mg/kg.

Results obtained from present study had shown that topical application of DMBA and croton oil produced 100% skin ulceration in carcinogen control group and decreased the oxidative stress parameters, i.e. reduced glutathione, SOD and catalase level to 3.3 ± 0.2 µmol/g, 1.7 ± 0.1 µmol/g and 13.6 ± 0.8 µmol of H_2O_2 reduction/mg protein/min respectively and increased the lipid peroxidation level as 7.7 ± 0.2 nmol/mg (Table II).

C. bonariensis (methanol) extract in 300 mg/kg caused reduced glutathione, superoxide dismutase and catalase increased up to 9.7 ± 0.7 µmol/g, 7.9 ± 0.6 µmol/g and 18.8 ± 0.7 µmol of H_2O_2 reduction/mg protein/min but lipid peroxidation decreased up to 4.2 ± 0.6 nmol/mg levels in the continuous group. While 600 mg/kg caused an induction in reduced glutathione 12.1 ± 1.1 µmol/g, superoxide dismutase 9.1 ± 0.8 µmol/g and catalase 25.5 ± 1.0 µmol of H_2O_2 reduction/mg protein/min and increased the lipid peroxidation level to 1.4 ± 0.4 nmol/mg as compared with the carcinogen control group ($p<0.001$) shown in Table II.

The phytochemical analysis showed the presence of reducing sugars, alkaloids, tannins, saponins, terpinoids, flavonoids, anthraquinones and glycosides and detection of quercetin by HPLC analysis is shown in Figure 3.

Discussion

Cancer chemoprevention by phytochemicals or herbal medicines is grabbing high interest now a day. These phytochemical exert their anti-cancer potential due to

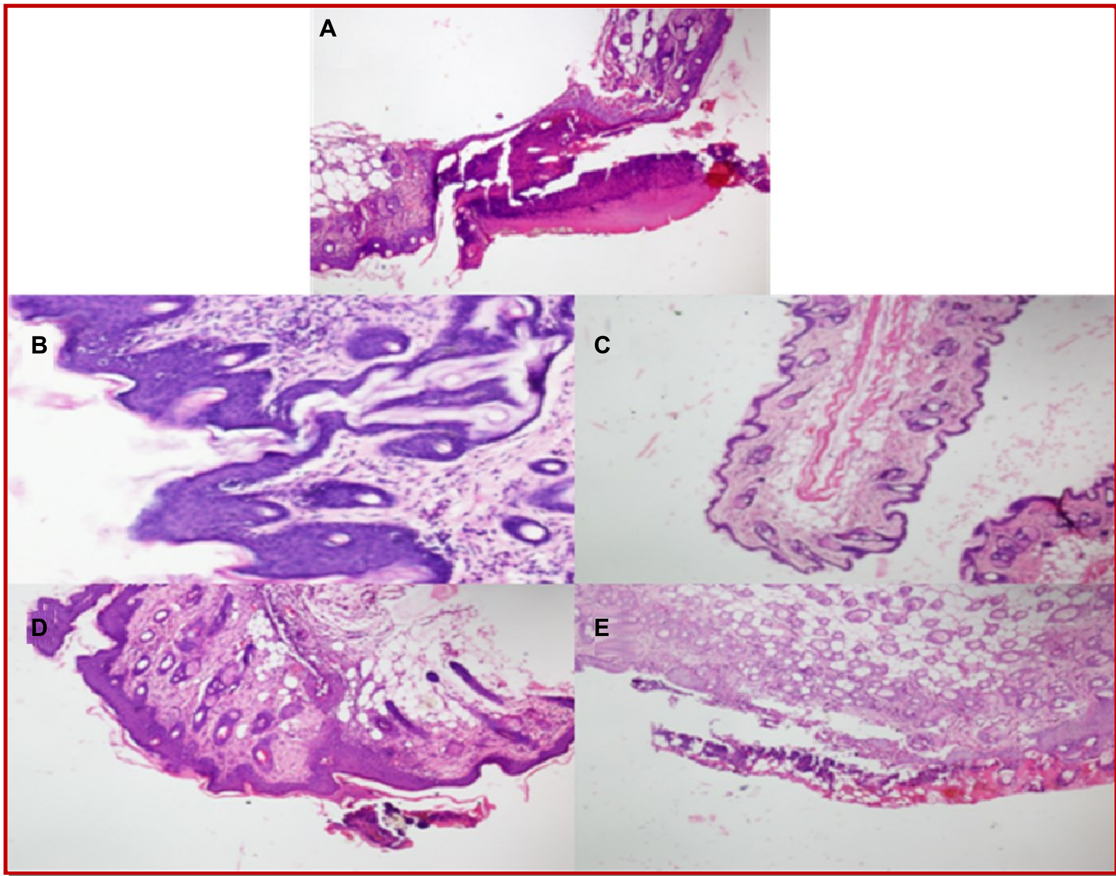

Figure 2: Histopathology of control and *C. bonariensis* (methanol) extract treated groups obtained at the end of study. Carcinogen Control (A) Ulceration and inflammatory slough on epidermis. *C. bonariensis* 300 mg/kg Pre group (B) Mild acanthosis, hyperkeratosis and mild papilomatosis with normal cytological features on epidermis. *C. bonariensis* 300 mg/kg Continuous group (C) Mild papillomatous changes on epidermis. *C. bonariensis* 600 mg/kg Pre group (D): Epidermis showed mild acanthosis with inflammation. *C. bonariensis* 600 mg/kg Continuous group (E) Mild degree of acanthosis with normal cytological features on epidermis

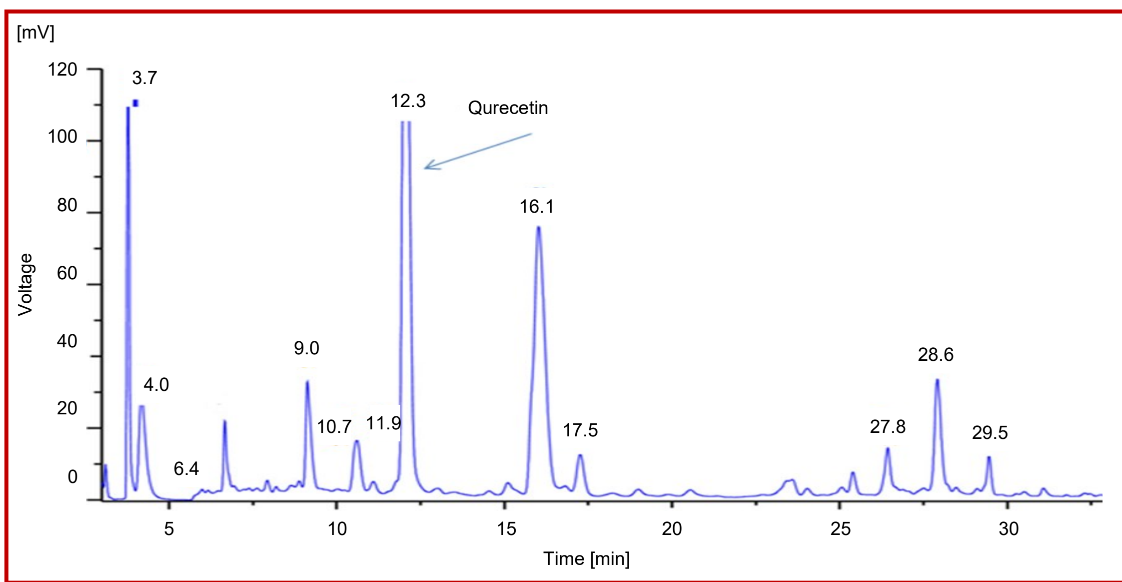

Figure 3: HPLC chromatogram for analysis of *C. bonariensis* methanol extract

chemical constituents such as flavonoids, polyphenols, carotenoids, terpinoids and tannins which have been obtained from our daily dietary agents. Flavonoids are potent anti-inflammatory, antioxidant and cytotoxic anti tumor agent. They have ability to reverse the process of carcinogenesis and inhibit the development of persistent tumor (Sengupta et al., 2004).

When tumor initiator, 7-12-dimethyl benz(a)anthera-cene (DMBA) and tumor promoter, croton oil (active constituent: 12-O-tetradecanoylphorbol-13-acetate) was applied on the mice skin, they produced inflammation and reactive oxygen species (ROS). These ROS including O_2-, OH-, H_2O_2 have ability to move from site of formation to the other healthy cells. DMBA with its active metabolites cause mutation in healthy cells via diol epoxide induction. Increased ROS disturb the balance of oxidation/reduction reaction, oxidative stress parameters and take part in chemical carcinogenesis by changing the gene expression and destructing the cellular components. TPA along with ROS, increase the epidermal ornithine decarboxylase, COX-2 and nitric oxide synthase level (Shakilur et al., 2008).

Similarly, enzymatic oxidative stress parameters including superoxide dismutase and catalase and non enzymatic reduced glutathione help to play important role in enzymatic defense system and their lower level promote the tumor in healthy cells. Reduced glutathione helps to protect the body from xenobiotics, toxic metabolites and ROS (Lu, 1999). Superoxide dismutase and CAT capture the reactive oxygen species and minimize their carcinogenic and mutagenic potential, balance the hydrogen/oxygen per oxide level by causing alteration in O_2 and H_2O_2 radical (Dasgupta et al., 2004). In carcinogen control group, level of reduced glutathione, superoxide dismutase and CAT were significantly decreased and lipid peroxidation increased along with the tumor incidence, tumor yield and tumor burden due to the presence of increased ROS.

C. bonariensis methanol extract decreased the tumor incidence, tumor yield, tumor burden, cumulative number of papilloma and lipid peroxidation level as compare to carcinogen control group. The plant extract increased the level reduced glutathione, superoxide dismutase and catalase in continuous group in which plant extract was applied throughout the experimental period (16 weeks) with higher effects at 600 mg/kg/b.wt as compare to 300 mg/kg. The phytochemical analysis had shown the presence of flavonoids, saponins, tannis and terpinoids and HPLC analysis indicated querectin i.e a flavonoid. It is a potent bioactive molecule that possess anticarcinogenic potential since it can interfere with the initiation, development and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis (Kumar et al., 2011). Flavonoids have potential as chemopreventive agent for cancer treatment due to their ability to induce apoptosis (Ramos, 2007) by arresting cell cycle at G1, S, G2 and M phases of cell cycle. Also, previously, it is known that quercetin has ability to capture the ROS, superoxide anions, hydroxyl and lipid peroxy radicals, inhibit cyclooxygenase, lipooxigenase, monoxygenase, phospholipase A2, protein kinase and NADH-oxidative pathways (Kumar et al., 2011).

Conclusion

C. bonariensis methanol extract possesses significant cytotoxic activity which may due to the presence of quercetin (a flavonoid) against DMBA-induced skin carcinogenesis.

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Conflict of Interest

Authors declare no conflict of interest

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